Am. J. Hum. Genet. 64:1045–1055, 1999

The "Thermolabile" Variant of Methylenetetrahydrofolate Reductase and Neural Tube Defects: An Evaluation of Genetic Risk and the Relative Importance of the Genotypes of the Embryo and the Mother

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Summary

Recent reports have implicated the "thermolabile" (T) variant of methylenetetrahydrofolate reductase (MTHFR) in the causation of folate-dependent neural tube defects (NTDs). We report herein the largest genetic study of NTD cases (n = 271) and families (n = 218) to date, establishing that, in Ireland, the "TT" genotype is found in 18.8% of cases versus 8.3% of controls (odds ratio 2.57; confidence interval [CI] 1.48–4.45; P = .0005). The maternal and paternal TT genotypes have intermediate frequencies of 13.8% and 11.9%, respectively, indicating that the predominant MTHFR-related genetic effect acts via the TT genotype of the developing embryo. Analysis of the 218 family triads of mother, father, and affected child with log-linear models supports this interpretation, providing significant evidence that the case TT genotype is associated with NTDs (P = .02) but no evidence of a maternal TT genotypic effect (P = .83). The log-linear model predicted that the risk of NTDs conferred by the case TT genotype is 1.61 (CI 1.06–2.46), consistent with the paramount importance of the case TT genotype in determining risk. There is no compelling evidence for more than a modest additional risk conferred by a maternal TT genotype. These results favor a biological model of MTHFR-related NTD pathogenesis in which suboptimal maternal folate status imposes biochemical stress on the developing embryo, a stress it is ill-equipped to tolerate if it has a TT genotype.

Received May 11, 1998; accepted for publication January 21, 1999; electronically published March 17, 1999.

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0002-9297/99/6404-0016$02.00

Introduction

Neural tube defects (NTDs), in particular spina bifida and anencephaly, are among the most common severe congenital malformations and represent a long-term public health burden in both social and economic terms (Centers for Disease Control 1989). Although the cause of NTDs remains poorly understood, inheritance of predisposing genetic factors is generally considered to be an important contributor (Little and Nevin 1992). Women who have already given birth to an affected child have a 10- to 15-fold increased risk that a subsequent child will have an NTD (Little 1992), suggesting that there is a strong maternal and/or familial pathogenic component. A series of studies over the past decade have established that folic acid supplements taken periconceptionally can greatly reduce (up to 72%) a woman's risk of both NTD occurrence and NTD recurrence in her offspring (MRC Vitamin Study Research Group 1991; Czeizel and Dudas 1992). Because most women carrying affected embryos have plasma and red blood cell folate levels well above the clinically deficient range (Kirke et al. 1993; Daly et al. 1995), it is apparent that folic acid does not act by correcting a simple severe nutritional deficiency. In the early 1990s, several studies established that women carrying embryos with NTDs (Kirke et al. 1993; Mills et al. 1995) and women who have given birth to affected infants (Steegers-Theunissen et al. 1991, 1994) have significantly elevated plasma levels of the amino acid homocysteine (i.e., they are hyperhomocysteinemic) and significantly reduced plasma folate and vitamin B_{12} concentrations. Of the five enzymes that control homocysteine levels—cystathionine β-synthase (CβS), betaine methyltransferase, S-adenosyl homocysteine hydrolase, methionine synthase (MS), and 5,10-methylenetetrahydrofolate reductase (MTHFR)—the latter two are folate dependent, and genetic variants thereof are obvious candidates as risk factors involved in the causation of folate-preventable NTDs. To date,

studies of the MS (Morrison et al. 1997; van der Put et al. 1997*b*) and CβS (Ramsbottom et al. 1997) genes have failed to identify any variants that are significantly associated with NTDs.

MTHFR regulates the methionine synthase–mediated remethylation of homocysteine to methionine by catalyzing the rate-limiting reduction of 5,10-methylenetetrahydrofolate to the methyl group donor 5-methyltetrahydrofolate (Scott and Weir 1994). Severe deficiency of the enzyme, which is critical in controlling basal concentrations of homocysteine, results in massively elevated plasma homocysteine concentrations and homocystinuria, a syndrome characterized by multiple physical, developmental, and cognitive defects (Rosenblatt 1989). Kang and colleagues (1988) identified a form of MTHFR, in ~5% of the white population, that is biochemically "thermolabile" in that its residual activity after incubation at 46°C for 5 min is significantly less than that of the wild-type enzyme. Individuals with the thermolabile enzyme were subsequently shown to be at higher risk for hyperhomocysteinemia (Engbersen et al. 1995). After MTHFR cDNA was cloned (Goyette et al. 1994), the mutation underlying the thermolabile isoform was identified as a C→T transition at nucleotide 677 (Frosst et al. 1995) that mandates the replacement of an alanine by a valine in a functionally important region of the molecule. Several recent studies have established that individuals with the thermolabile "TT" genotype are at greatly increased risk of being hyperhomocysteinemic, and those who have this phenotype have significantly reduced plasma folate levels (van der Put et al. 1995; Harmon et al. 1996; Jacques et al. 1996). On the basis of the findings of the above-mentioned studies, a consensus is emerging that TT homozygotes have aberrant folate metabolism and an increased requirement for this nutrient. Consequently, individuals with the TT genotype may be at particular risk of hyperhomocysteinemia and homocysteine-related diseases when their plasma folate concentrations are toward the low end of the normal range.

Given the prevalence of hyperhomocysteinemia and low folate status in mothers of offspring with NTDs, there are sound theoretical grounds to expect that the TT genotype would be overrepresented in individuals with NTDs and in mothers who have had a child with an NTD. We (Whitehead et al. 1995) and van der Put et al. (1995) independently reported that such was the case in the Irish and Dutch populations, respectively. Studies by Ou et al. (1996), Kirke et al. (1996), and Christensen et al. (1997) have supported this association; however, others (de Franchis et al. 1995; Papapetrou et al. 1996; Wilcken and Wang 1996; Mornet et al. 1997; Speer et al. 1997; Koch et al. 1998) have published negative studies and/or questioned the validity of the conclusion that the TT genotype confers risk of NTDs. Some of the latter authors have challenged the reliability of the data supporting a positive association, on the grounds that they were derived from studies that involved relatively few subjects and potentially poorly matched controls and thus were prone to sampling errors. They further suggested that the allele transmission disequilibrium test (TDT) for linkage (Spielman et al. 1993) is a more robust and appropriate method for validating genetic hypotheses, such as the association of the thermolabile variant of MTHFR with NTD risk, since it eliminates concerns regarding the selection of appropriately matched controls and the distortions of gene frequencies that may be produced by underlying ethnic heterogeneity and consequent population stratification.

Herein we report the largest genetic association study of NTDs to date and present evidence that supports our initial estimates (Whitehead et al. 1995) of the level of risk conferred by the case MTHFR TT genotype. In addition, we confirm the pathogenic involvement of the T allele by documenting a significant bias in its transmission from heterozygous parents to NTD offspring in 218 families. Furthermore, the size of the study population allowed us to test for evidence of interactions between maternal and case MTHFR genotypes and to definitively establish that the case TT genotype is the predominant genetic determinant of MTHFR-derived NTD risk.

Subjects and Methods

Study Population

Blood samples for genetic analysis were collected after informed consent was obtained from the following categories of subjects: 218 individuals (120 female and 98 male) born with spina bifida or encephalocele between 1956 and 1995 (mean age 16 years on January 1, 1996), for whom blood samples from both parents were available for genotyping; their mothers (n = 218); and their fathers (n = 218). In the three families for which a sample was available from two affected siblings, only that obtained from the older sibling was used in the analyses reported herein. Blood samples were also collected from an additional 53 individuals with NTDs (23 female and 30 male), for 31 of whom maternal samples were also obtained. Subjects were identified from the records of the Irish Association for Spina Bifida and Hydrocephalus, a support group for families with an affected member, and from a register of NTD births in the Dublin maternity hospitals between 1976 and 1987. Diagnosis of NTDs was based on clinical records. We are not able to accurately estimate the proportion of NTD cases in Ireland represented by these cases. However, as in other such studies, the method of case recruitment precluded the enrollment of embryos or fetuses lost in utero or

neonates who died soon after birth; the case population is therefore biased toward viable live births.

Controls

Blood samples were collected from a systematic group of 242 women at their first antenatal clinic visit at the Coombe Women's, Rotunda, or National Maternity hospitals in Dublin and have been described elsewhere (Kirke et al. 1996; Molloy et al. 1997, 1998).

Mutation Analysis

DNA from whole blood or stored frozen buffy coats was isolated, and MTHFR C677T genotyping was performed by PCR and allele-specific restriction digestion as described by Frosst et al. (1995). The normal and variant alleles give rise to diagnostic *Hin*fI fragments of 198 and 175 bp, respectively, when resolved on 2% agarose gels.

Statistical Analysis

Comparisons of the relative frequencies of both the T allele and the homozygous TT genotype between affected individuals, parents, and controls were performed by means of Fisher's exact test to assess significance. Because the major effect of the MTHFR locus on homocysteine levels is mediated by the recessive TT genotype, the primary tests were applied to this genotype, rather than to the T allele frequency or the frequencies of the other two genotypes.

Allele transmission tests compare the frequency of allele transmissions and nontransmissions from heterozygous parents and can be adapted to test a variety of genetic mechanisms (Schaid 1996). Comparisons of the frequencies of T and C allele transmissions to offspring with NTDs from heterozygous parents (the TDT test [Spielman et al. 1993]) were performed by means of an exact McNemar test. Since the principal risk-conferring genotype is considered to be the TT homozygote (i.e., the genetic mechanism is recessive), comparison of transmitted and nontransmitted alleles was also performed for the subset of families in which a TT outcome was possible (i.e., families with a CC parent were excluded from this subset). This permitted an assessment of T allele transmission bias that was restricted to transmission events from the "at risk" matings.

In the case of a fetal disorder in which maternal alleles may contribute directly to the condition under analysis, and therefore to the risk, parental alleles may not, in fact, be entirely appropriate controls. Weinberg et al. (1998) have recently presented a log-linear model that allows assessment of both fetal and maternal effects. Accordingly, we have adopted this as our principal approach to analyzing allele transmission within families. We fitted a recessive effect within their model, assuming Hardy-Weinberg equilibrium. The log-linear model of the fetal genotypic effect is equivalent (Weinberg et al. 1998) to the likelihood methods of Schaid and Sommer (1994). We fitted the log-linear model by use of the POISSON command of the general statistics package STATA, release 5.0 (Statacorp), assuming Hardy-Weinberg equilibrium. Thus, in accord with the method of Weinberg et al. (1998), the POISSON regression included an offset term to allow for the double exposure of the double heterozygote mating, a linear term equal to the number of alleles carried by mother and father, and optional modeled terms, including case TT homozygosity and maternal TT homozygosity. Relative risks and confidence intervals (CIs) were calculated directly from the model.

Results

Association of the TT Genotype with NTD Risk

A total of 271 individuals with NTDs were genotyped, of whom 18.8% (51) were TT homozygous, 43.9% (119) were CT heterozygous, and 37.3% (101) were CC homozygous. This compares with 8.3% (20) TT, 44.6% (108) CT, and 47.1% (114) CC genotypes in the 242 controls. The frequency of the T allele is significantly higher in affected individuals than in controls—that is, 40.6% compared with 30.6% (odds ratio [OR] 1.56; 95% CI 1.21–2.02; $P = .0007$). MTHFR genotypes clearly have a different distribution among individuals with NTDs than among controls ($P = .002$, χ^2, 2 df), reflecting a deficit of the CC genotype in patients as well as an excess of the TT genotype.

The biological hypothesis underlying the genetic involvement of MTHFR variants in NTD outcome emphasizes the role of the biochemically thermolabile MTHFR phenotype, which is the result of being homozygous (and not merely heterozygous) for the T allele. Consistent with this hypothesis, the TT genotype is even more strikingly associated with cases than controls (OR 2.57; 95% CI 1.48–4.45; $P = .0005$). Among individuals with NTDs, there is a nonsignificant increase in risk for CT heterozygotes compared with CC homozygotes (OR 1.22; 95% CI 0.8–1.8). Thermolabile TT homozygotes, in turn, have a significantly greater risk than CT heterozygotes (OR 2.33; 95% CI 1.3–4.3). Thus, these data indicate that the TT genotype is a risk factor for NTDs, and there is only a nonsignificant suggestion that the CT genotype may be marginally higher in risk than the CC genotype.

Allele Transmission

The 271 NTD patients included 218 for whom maternal and paternal DNA samples were also available. These 218 family triads allowed us to determine the

Table 1

Maternal, Paternal, and NTD Case Genotype Combinations in All Triads

		NTD Case			
Maternal	Paternal	CC	CT	TT	Total
CC	CC	36	...	...	36
CC	CT	21	17	...	38
CT	CC	18	22	...	40
CT	CT	7	25	19	51
CC	TT	...	6	...	6
TT	CC	...	7	...	7
TT	CT	...	6	14	20
CT	TT	...	12	5	17
TT	TT	...	...	3	3
Total		82	95	41	218
Percentage		37.6	43.6	18.8	100

frequencies of all possible mother-father-child MTHFR genotype combinations (table 1), and this, in turn, permitted us to investigate whether there is a bias in the transmission of the T allele from heterozygous parents. We applied log-linear models, which provide a powerful test of allele transmission (Weinberg et al. 1998), to the family data, assuming Hardy-Weinberg equilibrium (table 2). A significant risk of NTDs is conferred by the case TT genotype (OR 1.62; 95% CI 1.06–2.45; P = .024). The NTD risk conferred by being either CT or TT (i.e., reflecting the protective effect of the CC genotype) was only 1.08 (P = .65). Thus, analysis of the family data alone confirms a risk-conferring role for the TT genotype that does not merely reflect population stratification of case and control samples.

Simpler tests that directly compare frequencies of transmitted and nontransmitted alleles were applied to the 218 triads. These tests have the advantage that they are simple to calculate and are robust in the presence of Hardy-Weinberg disequilibrium; however, they have less statistical power than the log-linear tests. Two such tests were performed. The first was conventional TDT, which tests allele transmission from heterozygous parents regardless of potential case genotype outcome. Table 3 indicates that, from a total of 217 heterozygous parents, the T allele is transmitted 121 times (55.8%), whereas the C allele is transmitted only 96 times (44.2%). This bias in T allele transmission is only suggestive of an effect (P = .10). However, since our hypothesis is that TT is the major "at risk" genotype (i.e., the genetic mechanism is principally recessive), and that much less, if any, risk resides in the CT genotype, we do not believe the standard TDT is the most appropriate test to assess the importance of the *MTHFR* locus.

The second test was allele transmission from parents in families in which the parental genotypes would permit the offspring to have a TT genotype (i.e., each parent carries at least one T allele). Thus, only the fourth, seventh, and eighth matings (i.e., CT × CT, TT × CT, and CT × TT, respectively) listed in table 1 were used for this test, applying the standard TDT comparison of alleles within these matings alone. From the 139 heterozygous parents who could produce a TT offspring (in whom the risk-conferring recessive genotype under test could exert a pathogenic effect), the T allele is transmitted 82 times (59%), whereas the C allele is transmitted only 57 times (41%); this constitutes a bias toward T allele transmission that is statistically significant (P = .04). Schaid and Sommer (1994) proposed an alternative "REC" test for recessive loci; when applied to our data, this test shows a similar, though less significant, trend (P = .12).

Genotype Combinations in Families

The frequencies of the different mother-father-child MTHFR genotype combinations in the 218 triads also allowed us to assess the relative contribution of parental and case MTHFR genotypes to NTD risk. The highest TT genotype frequency, 18.8%, is observed in the affected offspring; TT homozygotes constitute 13.8% and 11.9% of maternal and paternal genotypes, respectively, compared with only 8.3% in controls (table 4). This suggests that it is the case TT genotype, rather than the maternal or paternal TT genotype, that is of paramount

Table 2

Log-Linear Models of Case and Maternal Effects within Parent-Child Triads

MTHFR Genotype	Relative Risk	P Value	95% CI	Overall χ^2	Estimated Allele Frequency
No effect	...	...	...	22.30	.37
Case TT	1.62	.024	1.06–2.45	17.42	.35
Case CT or TT	1.08	.654	.76–1.53	22.10	.37
Mother TT	.95	.814	.60–1.49	22.25	.38
Mother CT or TT	1.15	.422	.81–1.64	21.66	.37
Combined model:				17.37	.36
Case TT	1.61	.024	1.06–2.45	...	...
Mother TT	.95	.833	.60–1.50	...	...

Table 3

Allele Transmission Frequencies from Heterozygous Parents in Parent-Child Triads

Genotype of Parents (*n*)	Transmission Frequency (*n* [%])	
	C Allele	T Allele
All heterozygotes (217)	96 (44.2)	121 (55.8)
Heterozygotes whose matings allow TT outcome (139)	57 (41.0)	82 (59.0)

importance in driving NTD pathogenesis. In addition, the CT genotype frequencies in mothers (49.5%) and fathers (50.0%), but not in patients (43.6%), are higher than the CT frequency observed in controls (44.6%). This is compatible with the hypothesis that the CT genotype has little or no overall impact on NTD risk to the embryo and indicates that it is the enrichment of T alleles in both mothers and fathers per se (because of the frequency of TT homozygotes in the offspring rather than as a direct effect) that is the principal risk-conferring MTHFR-related genetic factor in parents.

Parental genotypic effects independent of case effects.—Recent reports establishing that the homocysteine and folate status of the mother has an impact on NTD outcome (Kirke et al. 1993; Daly et al. 1995; Molloy et al. 1998) have highlighted the need to verify whether the maternal MTHFR genotype has a direct effect on NTD risk. Although modeling of maternal effects in comparison with population controls is useful, it is subject to the criticism that the NTD families may be drawn from a subpopulation with a different underlying allele frequency. Accordingly, analysis should ideally be performed with the family data alone. However, the modeling of both maternal and case effects of genotypes within families is clearly complex. The log-linear model allows assessment of the risks conferred by maternal and case genotypes. Maternal TT genotype does not contribute significantly to the model ($P = .83$; OR 0.95; table 2). Neither does maternal CC have a significant protective effect ($P = .42$; table 2). Note that the maternal and case effects fitted within the model are independent of each other (Weinberg et al. 1998). Thus, the log-linear modeling indicates that a TT genotype in the embryo is the important MTHFR-mediated contributor to NTD outcome in the Irish population and provides no evidence in support of a maternal TT effect. It is worth noting that there is no maternal or paternal bias in the transmission of T alleles, because exactly the same number have been inherited from mothers ($n = 76$) as from fathers ($n = 76$) in those cases for which the parental origin of the T allele is unambiguous. This strongly suggests that parental genotypes do not play a role in NTD etiology through biased genomic imprinting of the MTHFR gene.

Inspection of table 1 reveals that, among matings in which one parent is TT and the other CT, there is an excess of TT outcomes when the mother is TT, but a deficit when the father is TT. This difference is statistically significant ($P = .02$). There is no obvious explanation for this disparity, because there is an excess of TT outcomes among double heterozygote matings, in which the mother is also a CT. It is unlikely to reflect a parent-specific segregation distortion, because approximately equal numbers of these reciprocal matings give rise to offspring with NTDs. Either this is merely a chance finding or the paternal TT genotype contributes functionally to NTD etiology—for example, through a complex imprinting mechanism. However, as outlined above, we could find no obvious evidence for such imprinting.

Mother-child genotype combinations.—Although the maternal TT genotype, in general, has no appreciable influence on NTD frequency as an independent risk factor, it could be important when other folate-related risk factors are present, including the TT genotype in the embryo. To establish whether the maternal genotype has any measurable effect on the NTD risk of TT embryos, we calculated the observed and expected frequencies of the 249 case TT–mother genotype combinations in our study (table 5).

Expected frequencies were calculated in two ways: The first method, "population expectation," makes no a priori assumption about the case TT frequency and uses the T and C allele frequencies of the control population to calculate the expected frequency of TT cases, and hence the expected case TT–mother TT and case TT–mother CT combinations. Thus, the expected frequency of the case TT–mother CT combination is $(0.306 \times 0.306) \times 0.694 = 6.50\%$, a figure that is computed by multiplying the expected frequency of the case TT genotype (i.e., the product of the population frequencies of its component alleles) by the population frequency of the mother's nontransmitted allele (in this case, C).

The second method, "conditional expectation," takes into account the observed elevation in case TT frequency and simply calculates the expected frequencies of possible case TT–mother genotype combinations by mul-

Table 4

Maternal, Paternal, and NTD Case Genotype Frequencies in All Parent-Child Triads

Individual	*n*	Genotype (%)		
		CC	CT	TT
NTD cases	218	37.6	43.6	18.8
NTD mothers	218	36.7	49.5	13.8
NTD fathers	218	38.1	50.0	11.9
Controls	242	47.1	44.6	8.3

Table 5

Observed and Expected Frequencies of Mother-Child Genotype Combinations

Genotype		Observed		Population Expectation			Conditional Expectation		
Mother	Child	Number	%	%	OR	95% CI	%	OR	95% CI
TT	TT	20	8.03	2.86	2.98	1.2–7.2	6.14	1.33	.7–2.7
CT	TT	30	12.05	6.50	1.97	1.0–3.7	13.94	.85	.5–1.4
TT	CT	15	6.02	6.50	.92	.4–1.9	6.45	.93	.5–1.9
CT	CT	73	25.30	21.24	1.26	.8–1.9	21.08	1.27	1.0–2.3
CC	CT	27	10.84	14.74	.70	.4–1.2	14.63	.71	.4–1.2
CT	CC	29	11.65	14.74	.76	.5–1.3	11.55	1.01	.6–1.7
CC	CC	65	26.10	33.43	.70	.5–1.0	26.20	1.00	.7–1.4
Total	…	249	100	100	…	…	100	…	…

tiplying the actual case TT frequency by the control population frequency of the nontransmitted maternal allele (i.e., 30.6% and 69.4% for the T and C alleles, respectively). Note that, in both of these methods, the population frequency of the maternal allele that is transmitted to the child is not included in the calculation, because it is the fixed sine qua non that defines the relationship under analysis, and therefore has a mandated frequency of 100%.

Applying these frequency calculations to all seven possible case-mother genotype combinations permitted relative risks for each to be calculated (table 5). By the population expectation method, which provides an estimate of the level of risk relative to the general population, the case TT–mother TT combination has a relative risk of 2.98, whereas there is a lower, but still significant, risk of 1.97 for case TT–mother CT combinations. These risk values are, respectively, higher and lower than that conferred to the affected child by the TT genotype when it is considered independently of the maternal genotype (OR = 2.57), the calculation of which was based on the observed genotype frequencies in the full 271-subject case group. To test whether these calculations reflect a significant excess of TT mothers of TT offspring (in comparison to CT mothers of TT offspring), we compared the frequency with which the nontransmitted allele in mothers of TT cases is a T allele (20 of 50 = 40.0%) with the T allele frequency in the control population (148 of 484 = 30.6%). This increased incidence (OR 1.51; 95% CI 0.83–2.75; P = .20) defines an excess of TT mothers and is therefore suggestive of additional risk in TT mother–TT embryo combinations, which is attributable to the maternal genotype but is not statistically significant. The risk estimates calculated by the conditional expectation method provide a more direct means of assessing the above, since the effect of the maternal genotype is isolated; consequently, any bias toward a particular maternal genotype would indicate that it confers a specific risk in addition to that conferred by the genotype of the embryo itself. From table 5it is clear that the excess of mothers of TT embryos who themselves have the TT genotype (OR 1.33; 95% CI 0.7–2.7) is again only suggestive and is also not statistically significant. Thus, our data do not support a strong maternal genotypic effect in the etiology of MTHFR-related NTDs.

Discussion

Recently, it has been proposed that a common, mildly dysfunctional "thermolabile" variant of the folate-dependent enzyme MTHFR is a genetic factor that accounts for at least some of the observed association of NTDs with low maternal folate status and high plasma homocysteine concentrations. Furthermore, the observation that individuals with the variant have an increased folate requirement may explain at least some of the prevention of NTD pregnancy outcome that can be achieved with periconceptional folic acid supplementation.

The homozygous thermolabile "TT" MTHFR genotype was first identified as a risk-conferring genotype in NTD pregnancy outcome by us (Whitehead et al. 1995) and by a group in The Netherlands (van der Put et al. 1995); further evidence in support of a pathogenic role for the TT genotype in NTD etiology was presented by Ou et al. (1996) and Christensen et al. (1997). A number of articles, however (de Franchis et al. 1995; Papapetrou et al. 1996; Wilcken and Wang 1996; Mornet et al. 1997; Speer et al. 1997; Koch et al. 1998), either reported a lack of association between the TT genotype and NTDs or challenged the validity of the studies that supported a positive association on the grounds that they were based on a case-control design that is inherently vulnerable to sampling error. Both de Franchis et al. (1995) and Wilcken and Wang 1996 cautioned that population frequencies of the TT genotype vary widely among white populations and that larger numbers of subjects would be required to make valid population-specific statements regarding the level of risk conferred by this genotype. The others also presented data that failed to show a statistically significant association of the TT genotype

with NTD outcome, regardless of whether analytical methods based on population frequencies or alternatives such as allele TDT were applied. The number of subjects recruited into each of these latter studies was, however, very small.

On the basis of the data presented here, we estimate the attributable fraction of NTDs due to the MTHFR TT genotype in Ireland at 11.4%, assuming a causal relationship between the TT genotype and these malformations, and by means of the formula of Kelsey et al. (1986) for computing attributable fraction. The highest estimate of population-attributable fraction in any study to date is 19% (Ou et al. 1996). Clearly the great majority of NTDs, including a majority of those that are folate preventable, must be driven by other, non–TT-related etiologic factors. As the vast majority of TT homozygotes do not have NTDs, it is also clear that the TT genotype is a risk factor with very low penetrance. It is, therefore, not surprising that studies involving very small numbers of patients have failed to show any association between TT genotype status and NTDs. Given these facts, it is apparent that large numbers of individuals with NTDs (and their parents) are needed for either genotype association studies or the allele TDT to have sufficient statistical power to enable definitive conclusions regarding the causative role of the TT genotype. The requisite numbers of subjects can be generated by performing larger "single-center" studies within genetically homogeneous populations or by the pooling of data from several independent association studies to perform a meta-analysis, such as that recently reported by van der Put et al. (1997*a*). We believe that the latter method is not an ideal solution to the need for large numbers of subjects for investigating the relationship between the TT genotype and NTDs, since it would draw cases and controls from ethnically distinct groups that are known to have very different MTHFR allele frequencies and would not be controlled for national or region-specific environmental factors, such as folate consumption, that are critical for evaluating genetic factors linked to nutrition. In addition, meta-analyses may be biased in favor of positive outcome, because negative studies are generally underreported in the literature.

The MTHFR genotype data presented herein are, to our knowledge, derived from the analysis of the largest number ($n = 271$) of NTD cases reported to date, and reinforce the hypothesis that the embryonic TT genotype is a significant genetic risk factor for NTD pregnancy outcome. The level of risk (OR 2.57; $P < .0005$) is lower than that reported by us in our initial study of 82 cases (OR 3.47) but is similar to that observed in a subsequent enlarged study of 153 cases (OR 2.61); each of these studies was based on a subset of the NTD cases analyzed here (Whitehead et al. 1995; Kirke et al. 1996). We therefore consider it to be a good estimate of the risk conferred by the TT genotype in the Irish population.

In establishing whether a genetic condition is, in fact, caused by a particular candidate allele, tests of allele transmission within families rather than case-control comparisons are considered by many to provide a better standard of proof. Such tests use the nontransmitted parental alleles as controls and thereby avoid the false conclusions that may result from genetic stratification between case and control populations. Three recent studies of T allele transmission to NTD offspring (Papapetrou et al. 1996; Speer et al. 1997; Koch et al. 1998) were based on small numbers—24, 58, and 70 heterozygous parents, respectively, who transmitted 12, 31, and 34 T alleles—and concluded that there is no evidence in support of a causative relationship between the T allele and NTD phenotype. In view of our contention that the TT genotype is a low-penetrance genetic factor acting in the context of a large majority of NTD cases that have arisen via other mechanisms, it is extremely unlikely that any study involving relatively small numbers of families, such as those reported in the literature thus far, could have generated a statistically significant result, either positive or negative, by using the TDT. Furthermore, the test has hitherto been applied to the transmission of the T allele from all heterozygous parents, apparently without consideration of the possible genotype outcome in the offspring, which is, of course, also dependent on the genotype of the other parent. Since the case genotype frequencies in our study established that TT, but not CT, is elevated relative to controls, the former is clearly the principal risk-conferring genotype. Thus, we postulate that the thermolabile MTHFR allele essentially acts in a recessive manner to generate a mildly dysfunctional phenotype in TT homozygous embryos that is potentially pathogenic when folate levels are low. This is consistent both with the biochemistry of the thermolabile variant and with our understanding of the pathophysiology of NTDs. We therefore believe that assessment of the genetic impact of the T allele in NTD causation requires that the TDT be used in a manner that takes into account the likely underlying biological mechanism. When we applied a modified TDT, which accommodates the proposed biochemical mechanism, to our data set, we observed a T allele transmission rate of 59%, a bias that is statistically significant ($P = .04$). In addition, log-linear models of a recessive effect demonstrated a significant role for the case TT genotype ($P = .02$). These results powerfully underscore the conclusion previously reached from association studies that the case TT genotype is a genetic risk factor for NTD outcome.

The TT genotype frequency in individuals with NTDs and their mothers can be used to establish the relative importance of the case and maternal genotypes to the

cause of NTDs. In our 218 triads, the case and maternal TT frequencies are 18.8% and 13.8%, respectively, compared with 8.3% in the controls. It is clear from these figures that MTHFR-associated NTD pathogenesis in the Irish population is principally dependent on the TT genotype status of the embryo rather than the mother. Log-linear modeling confirmed that the maternal TT genotype is not a significant independent contributor to NTD risk ($P = .83$). This conclusion is contrary to the conclusion that would be reached by considering the data reported by van der Put et al. (1995), in which the case and maternal TT frequencies were 13% and 16%, respectively. This Dutch study, however, was based on only 55 case and 70 maternal genotypes, and it is unclear how many of these constituted mother–affected offspring pairs. In a subsequent report from the same group using only 51 such pairs (van der Put et al. 1996), the frequency of TT homozygotes was even more skewed, 19.6% TT in mothers and 13.7% TT in cases. There are two possible explanations for this apparent discrepancy. It is possible, but perhaps unlikely, that the biological mechanism leading to a failure to close the neural tube differs between the populations in Ireland and The Netherlands because of additional, as yet undefined, nutritional and/or genetic factors, specific to the latter country, that act through the mother. Alternatively, the small sample size in the Dutch study may have produced an excess of the TT genotype in mothers relative to that observed in cases, either by chance or because of a disproportionate loss of TT embryos prior to birth. In Ireland, the prevailing legal and social constraints on prenatal screening and elective abortion are such that it is unlikely that our case population has been subjected to genotype-specific preterm loss through medical intervention. We therefore believe that our data provide strong evidence that the embryonic TT genotype is of paramount importance in determining NTD outcome.

The question of the extent, if any, to which the maternal MTHFR TT genotype actively contributes to NTD outcome could also be addressed with the large number ($n = 249$) of mother–NTD case pairs in our study. Hyperhomocysteinemia is a maternal phenotype that has been associated with NTD outcome (Mills et al. 1995), and there is good evidence from genotype-nutrition interaction studies that TT homozygotes are at risk of hyperhomocysteinemia when plasma folate concentrations are low and that the thermolabile MTHFR enzyme may further depress plasma folate levels that are already low (Harmon et al. 1996; Jacques et al. 1996). A plausible biological hypothesis exists, therefore, to explain how the maternal TT genotype could play a particular pathogenic role in NTDs, either by limiting the supply of folate to the embryo or by facilitating the accumulation and increased transfer to the embryo of homocysteine, high concentrations of which can disrupt neural tube closure in experimental models (Rosenquist et al. 1996). However, it is also plausible that the pathogenic impact of the TT genotype in the context of low folate is exclusive to the embryo (see above discussion) and therefore that hyperhomocysteinemia (and the TT genotype) is more prevalent in women who give birth to a child with an NTD merely because the excess number of TT homozygotes in the NTD offspring drives up the frequency of the T allele, and TT homozygotes, in the mothers.

We have used two methods to calculate the expected frequencies of the various mother–affected offspring genotype combinations, an approach that is based on control C and T allele frequencies and is adjusted to allow for the fact that the mother inevitably shares one T allele with her child. These have been compared to the observed frequencies of the genotype combinations. Our data, derived from a population expectation method, indicate that the risk conferred by a TT mother–TT child combination (OR 2.98) is greater than that conferred by a CT mother–TT child combination (OR 1.97). However, the difference between the combinations is not statistically significant. In a recent report, van der Put et al. (1996) claimed that the frequency of the maternal TT–case TT genotype combination was significantly higher than expected (5.9% observed compared with 0.9% predicted from control allele frequencies) in a group comprising 51 Dutch mother–NTD child pairs. This claim, however, was based on only three such genotype combinations identified within a study population in which the proportion of TT mothers relative to TT offspring is unexpectedly high. The analytical method used by van der Put et al. (1996), which was not clearly described in their report, also underestimated the expected frequencies of the rarer genotype combinations, especially the maternal TT–case TT combination (probably due, in part, to the use of control TT frequencies that are below Hardy-Weinberg predictions), and consequently leads to inflation of the risk estimates for these combinations. Recalculation of the data published by van der Put et al. 1996 by our population expectation method yields an expected maternal TT–case TT frequency of 1.68%, given the Dutch control T allele frequency of 25.6%, rather than the expected frequency of 0.9% reported; as a result, the OR based on the very small data set from the Dutch group fell from 6.1 to 3.67 and was no longer significant (95% CI 0.76–17.74). When we used our conditional expectation method (which is a better indicator of the potential maternal contribution) to recalculate the Dutch data, the expected frequency of the maternal TT–case TT combination was 3.51%, and the OR was even lower and less significant (OR 1.78; 95% CI 0.44–7.16)

We applied several other analytical methods to our data set, all of which yielded nonsignificant indications

that the maternal genotype plays, at most, a modest role in NTD pathogenesis. We believe that this issue can only be resolved either by studies of much larger numbers of mother–child pairs or by the application of the TDT to three-generation families that include heterozygous maternal grandparents of NTD offspring (in which being a mother of an NTD child is the condition under analysis), an approach that has been suggested by others (Mitchell 1997).

On the basis of the evidence presented here, it is clearly the TT genotype status of the developing embryo, rather than the TT genotype status of its mother, that is the critical genetic determinant of MTHFR-related NTD risk. Our data do not exclude the possibility that the maternal TT genotype may confer an additional risk to a TT embryo over and above the risk that can be attributed to the genotype of the embryo itself, but the effect of this is probably comparatively small. Our own previous work has indicated both that the maternal TT genotype is associated with lower red blood cell folate and that lower red blood cell folate increases NTD risk (Daly et al. 1995; Molloy et al. 1997). However, we have also established that plasma and red blood cell folate levels tend to be reduced in women who give birth to NTD offspring, regardless of MTHFR genotype (Molloy et al. 1998). Therefore, the maternal TT genotype might become only a modest risk factor by further depressing maternal folate levels that are already below a nominal threshold at which risk is enhanced. If such is the case, it is likely to be relevant in only a small proportion of NTD-affected pregnancies. Indeed, of the 50 TT offspring in this study for whom maternal MTHFR genotypes are known, 20 have TT mothers, rather than the expected 15; a simplistic evaluation would therefore conclude that only 5 (10%) of these offspring have an NTD that may be attributable to additional risk conferred by the maternal TT genotype. At present, we favor a biological model of MTHFR-driven NTD causation in which the mother's folate status, which may be mildly compromised by having a TT genotype, determines the exposure of the embryo to pathogenic challenges that it is particularly ill-equipped to overcome if it is a TT homozygote expressing the thermolabile form of the enzyme.

In summary, this study provides compelling evidence that the MTHFR TT genotype is a risk factor for NTDs, at least in populations in which folate nutrition is suboptimal. We estimate that the population-attributable fraction in Ireland is ~12%. Although the majority of NTDs must, therefore, be caused by other genetic and/or environmental factors, we nevertheless achieved statistical significance with methods based on comparisons with population controls and with methods based on log-linear modeling and allele transmission biases within families. Furthermore, we have established that the case TT genotype is the principal determinant of MTHFR-related NTD outcome and that the maternal TT genotype plays, at most, a minor role. The population-attributable fraction may be <12% in countries that have a higher dietary or supplemental folate/folic acid intake than Ireland, but it is likely that the TT genotype is a universal genetic risk factor for embryos under low folate conditions. Future studies should be directed toward defining the folate threshold below which the TT genotype becomes potentially pathogenic, and public health policy should incorporate directed or general folic acid supplementation strategies that are designed to prevent folate levels in at-risk pregnant women from falling below that threshold.

Acknowledgments

We are grateful to the families who participated in this study, and thank the Irish Association for Spina Bifida and Hydrocephalus and the Coombe, National Maternity, and Rotunda hospitals for making possible the collection of samples. We thank Dr. Laura Mitchell for her helpful suggestions regarding data analysis. The Health Research Board, Ireland, and the National Institute of Child Health and Human Development, U.S.A., provided financial support.

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